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(54) Enzymatic process for the preparation of L-cysteine and L-cystine

(57) L-Cysteine and/or L-cystine may be prepared by reacting a β-substituted L-alanine represented by the general formula (I):

$$X-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}COOH \qquad (I)$$

wherein X is a halogen atom, or an –OR or –SR group where R is a hydrogen atom, or an alkyl, acetyl, benzyl or sulfonic acid group, with a metal sulfide, a metal hydrosulfide, a metal polysulfide, ammonium sulfide, ammonium hydrosulfide or an ammonium polysulfide in the presence of tryptophan synthase.

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SPECIFICATION

Enzymatic process for preparing sulfur containing L-amino acids

Field of the Invention

The present invention relates to a process for preparing L-cysteine and/or L-cystine by reacting a β-substituted L-alanine with a metal sulfide, a metal hydrosulfide, a metal polysulfide, ammonium sulfide, ammonium hydrosulfide or an ammonium polysulfide in the presence of tryptophan synthase.

L-Cysteine and L-cystine are widely utilized for cosmetic applications, in the food industry as a food additive, and for other applications, as well as for medical applications, for example, as a constituent of transfusions.

Prior Art

Various processes for preparing L-cysteine and L-cystine have hitherto known: typically, (1) extraction from natural materials such as hair, (2) chemical syntheses as disclosed in, for example, Japanese Patent Application Laying-open No. SHO 57–200356, (3) an enzymatic synthesis from DL-2-aminothiazoline-4-carboxylic acid as disclosed in Japanese Patent Publication No. SHO 54–2272, and (4) a reaction of a β-substituted alanine with a metal sulfide or hydrosulfide in the presence of cysteine desulfhydrase as disclosed in Japanese Patent Publication No. SHO 57–21311 are known. However, these are not necessarily advantageous for an industrial process.

Summary of the Invention

Considering such status of the art, the present inventors have studied a new process for preparing L-cysteine and/or L-cystine at low cost and found that L-cysteine and/or L-cystine may be produced by a reaction of a β-substituted L-alanine with a metal sulfide, a metal hydrosulfide, a metal polysulfide, ammonium sulfide, ammonium hydrosulfide or an ammonium polysulfide in the presence of tryptophan synthase. Thus, we have attained this invention on the basis of this discovery.

Description of the Invention

Tryptophan synthase is known to exist widely in microorganisms, higher plants and others: refer to, for example, Bacteriological Reviews, Vol. 39, No. 2, pp. 87–120 (1975).

In the invention, tryptophan synthase derived from microorganisms is usually used but the enzyme source is not limited to this. Strains producing tryptophan synthase include, for example, *Escherichia coli* MT-10232 (FERM BP-19), *Escherichia coli* MT-10242 (FERM BP-20), *Neurospora crassa* ATCC-14692 and *Saccharomyces cerevisiae* ATCC-26787.

Extraction methods of tryptophan synthase from cultured cells are known and described in The Journal of Biological Chemistry, Vol. 249, No. 24, pp. 7756–7763 (1974) for *Ze. coli*; *ibid.*, Vol. 250, No. 8, pp. 2941–2946 (1975) for *Neurospora crassa*; and European Journal of Biochemistry, Vol. 102, pp. 159–165 (1979) for *Saccharomyces cerevisiae*; respectively.

Tryptophan synthase used in the invention is not necessarily an extracted and purified enzyme. Cultures of a tryptophan synthase producing microorganism, live cells collected from the cultures by centrifugation or a similar method, dried cells thereof, and cell debris obtained by grinding, ultrasonicating or otherwise treating the cells or by autolysis thereof may be utilized as an enzyme source in the invention. Extracts from these cells and crude enzymes obtained from the extracts may also be utilized. Further, immobilized cells or enzymes may of course be utilized in the invention.

Any synthetic or natural medium may be used for the culture of tryptophan synthase producing cells, provided that it contains a carbon source, a nitrogen source, minerals and, optionally, a small amount of minor nutrient(s). Addition of a small amount of tryptophan or indole to the culture medium may sometimes be effective. Also, the amount of tryptophan synthase produced may sometimes be increased by adding a small amount of indoleacrylic acid to the medium.

The culture is aerobically carried out by shaking or aeration agitating culture. The temperature is in the range of 20 to 40°C, usually 25 to 37°C. The pH of the culture medium is 5 to 8.

It is well known that tryptophan synthase is a multifunctional enzyme, that is, the enzyme catalyzes not only the synthesis of L-tryptophan from indole-3-glycero-phosphoric acid and L-serine but also many other reactions: refer to, for example, Advances in Enzymology and Related Areas of Molecular Biology, Vol. 49, pp. 127–185 (1979).

However, the present inventors have for the first time discovered that the reaction according to the invention may be catalyzed by tryptophan synthase.

β-Substituted L-alanines, one of the substrates for the reaction, which may be used in the invention include, for example, β-halogeno-L-alanine, such as β-chloro-L-alanine and β-bromo-L-

such as S-methyl-L-cysteine and S-ethyl-L-cysteine; O-acetyl-L-serine; O-benzyl-L-serine; S-benzyl-L-cysteine; L-serine O-sulfate; L-serine; and the like.

Sulfides, hydrosulfides or polysulfides, the other substrate, which may be used in the invention include, for example, metal sulfides, such as sodium sulfide, potassium sulfide and lithium sulfide; metal hydrosulfides, such as sodium hydrosulfide, potassium hydrosulfide and lithium hydrosulfide; metal polysulfides, such as sodium polysulfides and potassium polysulfides; ammonium sulfide; ammonium hydrosulfide; ammonium polysulfides; and the like.

According to the invention, the β-substituted L-alanine and the sulfide, hydrosulfide or polysulfide are usually reacted in an aqueous medium at a pH of 6 to 10 in the presence of tryptophan synthase. The reaction temperature is suitably selected from the range of 20 to 60°C. The reaction period of time is generally 1 to 100 hours for a batch reaction although it may vary depending on the titer of the enzyme, the concentrations of the substrates used and other conditions. The reaction is carried out stationarily or under slow agitation.

The concentrations of the substrates, the β-substituted L-alanine and the sulfide, hydrosulfide or polysulfide, are not particularly limited but usually in the range of about 0.1–30% by weight. The substrates may be added wholly at one time when the reaction is started or, alternatively, they may be added partially as the reaction proceeds. Desirably, the sulfide, hydrosulfide or polysulfide is present in the reaction medium at an equimolar amount or more based on the β-substituted L-alanine. In the reaction, it is desirable to add a small amount of a coenzyme, pyridoxal phosphate, in addition to the substrates.

When cells or culture media of a microorganism capable of producing tryptophan synthase are utilized as an enzyme source, the yield may be increased by adding at least one compound selected from alcohols, esters, ketones and surfactants to the reaction medium. The alcohols which may be used include ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutyl alcohol, tert-butyl alcohol, 1-pentanol, 1-octanol and the like. The esters which may be used include ethyl formate, propyl formate, butyl formate, ethyl acetate, propyl acetate, butyl acetate, isobutyl acetate and the like. The ketones which may be used include acetone, methyl ethyl ketone, methyl isobutyl ketone and the like. The surfactants which may be used include anionic surfactants, such as sodium dodecyl sulfate and sodium deoxycholate, nonionic surfactants, such as octyl phenyl ethers, and other surfactants. The amount of these compounds added to the reaction medium is usually in the range of 0.01–5% by weight although it may vary with the type of the compounds or the strain used.

When the reaction is thus carried out, the reaction medium will generally contain a mixture of L-cysteine and L-cystine since L-cysteine produced is readily oxidized and converted to L-cystine. The amount of L-cystine gradually increases as the reaction proceeds. However, the ratio of the concentration of L-cysteine to that of L-cystine can be varied by controlling the reaction conditions.

L-Cysteine or L-cystine may be recovered from the reaction medium in any conventional manner. For example, L-cystine, which is difficultly soluble in water, can readily be isolated by aerating the reaction medium to oxidize a major proportion of L-cysteine to L-cystine after the completion of the reaction. L-cysteine may be obtained by subjecting the thus obtained L-cystine to electrolytic reduction.

Quantitative measurement of L-cysteine and L-cystine was carried out by liquid chromatography. Further, liquid chromatography using a column for separating optical isomers showed that both the cysteine and cystine produced had the L-configuration.

Description of the Preferred Embodiments

The invention will hereinbelow be illustrated by the following examples.

Example 1

Escherichia coli MT-10242 (FERM BP-20) was inoculated in a liquid medium of 1% meat extract, 0.5% peptone, 0.1% yeast extract and 0.2% KH_2PO_4, pH 7.0, and cultured with shaking at 30°C for 20 hours. After the culture, cells were collected by centrifugation and the enzyme was purified according to the method of O. Adachi *et al.*, The Journal of Biological Chemistry, Vol. 249, No. 24, pp. 7756–7763 (1974). The thus obtained enzyme tryptophan synthase having a specific activity (titer) of 9.2 units per mg was used in the following reaction. The enzyme activity was determined by the method of C. Yanofsky *et al.*, Methods in Enzymology, Vol. 15, pp. 801–807 (1962). One unit is represented by the amount of the enzyme producing 1 μmol/min of tryptophan from L-serine and indole at 37°C, pH 7.8.

Tryptophan synthase, 0.5 mg, was added to 10 ml of a reaction medium, pH 8.5, containing 50 mM L-serine, 100 mM of a sulfide, hydrosulfide or polysulfide indicated in Table 1 and 0.1 mM pyridoxal phosphate, and shaked slowly at 35°C for 10 hours. The concentrations of L-cysteine and L-cystine produced and the yields of the total thereof based on L-serine are shown in Table 1.

Table 1

Kind of Sulfides etc.	L-cysteine (mM)	L-cystine (mM)	Yield (mole %)
Sodium sulfide	18	6.5	62
Sodium hydrosulfide	16	5	52
Potassium sulfide	11	3.5	36
Ammonium sulfide	8	1.5	22
Ammonium hydrosulfide	6	2	20
Ammonium trisulfide	6	1.5	18
Sodium disulfide	11	3	34

Example 2

According to the method of W. H. Matchett *et al.*, The Journal of Biological Chemistry, Vol. 250, No. 8, pp. 2941–2946 (1975), *Neurospora crassa* ATCC-14692 was cultured and the enzyme was purified. The thus obtained enzyme tryptophan synthase had a specific activity of 1.3 units per mg.

The enzyme liquid was used in the following reaction. A reaction medium (pH 8.5, 10 ml) containing 50 mM L-serine, 100 mM sodium hydrosulfide, 0.1 mM pyridoxal phosphate and 1.3 units tryptophan synthase was gently shaked at 35°C for 10 hours. There were produced in the reaction medium 12 mM L-cysteine and 4 mM L-cystine.

Example 3

Saccharomyces cerevisiae ATCC-26787 was inoculated in a liquid medium of 1% peptone, 0.5% yeast extract, 2% glucose and 0.01% indoleacrylic acid, pH 6.0, and cultured with shaking at 30°C for 20 hours. After the culture cells were collected by centrifugation. The enzyme was purified according to the method of M. Dettwiler *et al.*, European Journal of Biochemistry, Vol. 102, pp. 159–165 (1979). Thus, tryptophan synthase with a specific activity of 1.2 units per mg was obtained.

The enzyme liquid was used to effect the following reaction. A reaction liquid (10 ml) containing 50 mM L-serine, 100 mM sodium hydrosulfide, 0.1 mM pyridoxal phosphate and 1.2 units tryptophan synthase, pH 8.5, was gently shaked at 35°C for 10 hours. The resultant reaction liquid contained 11. mM L-cysteine and 3 mM L-cystine produced.

Example 4

Escherichia coli MT-10242 (FERM BP-20) was inoculated in a liquid medium of pH 7.0 containing 1% meat extract, 0.5% peptone, 0.1% yeast extract and 0.2% KH_2PO_4 and shaked at 30°C for 20 hours. After the culture, cells were collected by centrifugation and used as an enzyme source of tryptophan synthase. The wet cells had a specific activity of 120 units per g.

A reaction medium (100 ml) containing 200 mM L-serine, 300 mM sodium sulfide, 0.1 mM pyridoxal phosphate and 5 g of the wet cells and adjusted by HCl to pH of 8.5 was shaked at 35°C for 24 hours. After the reaction was completed, L-cystine present in the reaction medium was reduced to L-cysteine by dithiothreitol. The amount of L-cysteine produced was 98 mM.

Example 5

Tryptophan synthase obtained in Example 1 was used in the following reaction.

The tryptophan synthase (500 units) was added to 100 ml of a reaction liquid containing 300 mM of a β-substituted L-alanine indicated in Table 2, 600 mM sodium hydrosulfide, 1 mM pyridoxal phosphate and 480 mM tris-aminomethane-HCl buffer, pH 8.5, and gently shaked at 35°C for 1 hour. After the reaction was completed, L-cystine present in the reaction liquid was reduced to L-cysteine by dithiothreitol and then the amount of L-cysteine was measured. The results are shown in Table 2.

Table 2

β-Substituted L-alanine	L-cysteine (mM)
β-Chloro-L-alanine	82
O-Methyl-L-serine	116
O-Acetyl-L-serine	95
O-Benzyl-L-serine	24
S-Methyl-L-cysteine	27
S-Ethyl-L-cysteine	18
S-Benzyl-L-cysteine	5
L-Serine O-sulfate	37
L-Serine	123

Example 6

Escherichia coli MT-10232 (FERM BP-19) was inoculated in a liquid medium of pH 7.0 containing 1% meat extract, 0.5% peptone, 0.1% yeast extract and 0.2% KH_2PO_4 and shaked to culture at 30°C for 20 hours. After the culture, cells were collected by centrifugation and freezed at −20°C to store as an enzyme source of tryptophan synthase. The wet cells had a tryptophan synthase activity of 89 units per g. The stored cells were used in the following reaction.

A reaction medium (100 ml) containing 200 mM L-serine, 300 mM sodium sulfide, 0.1 mM pyridoxal phosphate, 100 mM trisaminomethane-HCl buffer (pH 8.5) and 5 g of the wet cells was gently shaked at 35°C for 10 hours. After the reaction was completed, L-cystine present in the reaction medium was reduced to L-cysteine by dithiothreitol and the amount of L-cysteine produced was then measured. The amount was 114 mM.

Example 7

The wet cells obtained in Example 6 were used in the following reaction.

A reaction liquid (100 ml) of pH 8.0 containing 200 mM (2.1 g) L-serine, 1000 mM sodium hydrosulfide, 0.1 mM pyridoxal phosphate and 5 g of the wet cells was gently stirred at 35°C for 10 hours. L-serine was further added at the time of 2 hours and 5 hours after the reaction was started, each in an amount of 2.1 g. During the reaction, the pH of the reaction liquid was maintained at 8.0 by adding 6N phosphoric acid. When the reaction was completed, 4.3 g of L-cysteine and 1.1g of L-cystine were accumulated in the reaction liquid.

Example 8

The wet cells obtained in Example 4 were used directly as an enzyme source of tryptophan synthase in the following reaction.

A reaction medium (100 ml, pH 8.5) containing 200 mM L-serine, 300 mM sodium sulfide, 1 mM pyridoxal phosphate, 5 g of the wet cells and a compound indicated in Table 3 was gently shaked at 35°C for 5 hours. The pH of the medium was controlled to 8.5 ± 0.3 during the reaction. After the reaction was completed, L-cystine present in the reaction medium was reduced to L-cysteine by dithiothreitol and the amount of L-cysteine produced was then measured. The results are shown in Table 3.

Table 3

Compound added (Concentration g/l)		Amount of L-cysteine (mM)
Control	(0)	67.2
Ethanol	(10)	99.6
1-Propanol	(10)	119.4
2-Propanol	(10)	105.6
1-Butanol	(10)	140.0
2-Butanol	(10)	128.7
Isobutyl alcohol	(5)	114.8
Tert-butyl alcohol	(5)	107.2
1-Pentanol	(5)	106.5
1-Octanol	(10)	128.7
Ethyl formate	(5)	96.7
Propyl formate	(5)	99.8
Butyl formate	(5)	104.3
Methyl acetate	(5)	98.4
Ethyl acetate	(5)	108.9
Butyl acetate	(5)	162.5
Isobutyl acetate	(5)	129.1
Acetone	(5)	96.5
Methyl ethyl ketone	(5)	111.8
Methyl isobutyl ketone	(5)	167.0
Sodium deoxycholate	(1)	139.4
Sodium dodecyl sulfate	(1)	160.9
Triton X-100	(1)	160.7

CLAIMS

1. A process for preparing L-cysteine and/or L-cystine comprising reacting a β-substituted L-alanine represented by the general formula (I):

$$X\text{–}CH_2\text{–}\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH \qquad (I)$$

wherein X denotes a halogen atom or an –OR or –SR group where R is a hydrogen atom or an alkyl, acetyl, benzyl or sulfonic acid group, with a metal sulfide, a metal hydrosulfide, a metal polysulfide, ammonium sulfide, ammonium hydrosulfide or an ammonium polysulfide in the presence of tryptophan synthase.

2. A process for preparing L-cysteine and/or L-cystine according to claim 1, wherein the reaction is carried out in the presence of at least one of 0.01 to 5% by weight selected from the group consisting of alcohols, esters, ketones, and surfactants.

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